Effect of fixation on brain and lymphoreticular vCJD prions and bioassay of key positive specimens from a retrospective vCJD prevalence study

Journal of Pathology
J Pathol 2011; **223:** 511–518
Published online 10 December 2010 in Wiley Online Library (wileyonlinelibrary.com) DOI: 10.1002/path.2821

ORIGINAL PAPER

Effect of fixation on brain and lymphoreticular vCJD prions and bioassay of key positive specimens from a retrospective vCJD prevalence study

Jonathan DF Wadsworth,[1] Inmaculada Dalmau-Mena,[1] Susan Joiner,[1] Jacqueline M Linehan,[1] Catherine O'Malley,[1] Caroline Powell,[1] Sebastian Brandner,[1] Emmanuel A Asante,[1] James W Ironside,[2] David A Hilton[3] and John Collinge[1]*

[1] *MRC Prion Unit and Department of Neurodegenerative Disease, UCL Institute of Neurology, National Hospital for Neurology and Neurosurgery, Queen Square, London WC1N 3BG, UK*
[2] *National CJD Surveillance Unit, University of Edinburgh, Bryan Matthews Building, Western General Hospital, Edinburgh EH12 6UW, UK*
[3] *Department of Histopathology, Derriford Hospital, Plymouth, PL6 8DSH, UK*

**Correspondence to: Professor John Collinge, MRC Prion Unit and Department of Neurodegenerative Disease, UCL Institute of Neurology, National Hospital for Neurology and Neurosurgery, Queen Square, London WC1N 3BG, UK e-mail: j.collinge@prion.ucl.ac.uk*

Abstract

Anonymous screening of lymphoreticular tissues removed during routine surgery has been applied to estimate the UK population prevalence of asymptomatic vCJD prion infection. The retrospective study of Hilton *et al* (*J Pathol* 2004; 203: 733–739) found accumulation of abnormal prion protein in three formalin-fixed appendix specimens. This led to an estimated UK prevalence of vCJD infection of ~1 in 4000, which remains the key evidence supporting current risk reduction measures to reduce iatrogenic transmission of vCJD prions in the UK. Confirmatory testing of these positives has been hampered by the inability to perform immunoblotting of formalin-fixed tissue. Animal transmission studies offer the potential for 'gold standard' confirmatory testing but are limited by both transmission barrier effects and known effects of fixation on scrapie prion titre in experimental models. Here we report the effects of fixation on brain and lymphoreticular human vCJD prions and comparative bioassay of two of the three prevalence study formalin-fixed, paraffin-embedded (FFPE) appendix specimens using transgenic mice expressing human prion protein (PrP). While transgenic mice expressing human PrP 129M readily reported vCJD prion infection after inoculation with frozen vCJD brain or appendix, and also FFPE vCJD brain, no infectivity was detected in FFPE vCJD spleen. No prion transmission was observed from either of the FFPE appendix specimens. The absence of detectable infectivity in fixed, known positive vCJD lymphoreticular tissue precludes interpreting negative transmissions from vCJD prevalence study appendix specimens. In this context, the Hilton *et al* study should continue to inform risk assessment pending the outcome of larger-scale studies on discarded surgical tissues and autopsy samples.

Keywords: variant Creutzfeldt–Jakob disease; prion disease; prion strain; prion transmission; bovine spongiform encephalopathy

Received 14 September 2010; Revised 21 October 2010; Accepted 6 November 2010

Conflicts of interest statement: JC is a Director and JC and JDFW are shareholders and consultants of D-Gen Limited, an academic spin-out company working in the field of prion disease diagnosis, decontamination, and therapeutics. D-Gen markets the ICSM35 antibody used in this study. SB carries out a large-scale screening study for the detection of abnormal prion protein in archival appendix samples. This study was commissioned in a competitive tender by the UK Health Protection Agency. The other authors declare no conflicts of interest..

Introduction

Experimental confirmation that variant Creutzfeldt–Jakob disease (vCJD) [1] is caused by the same prion strain as that causing bovine spongiform encephalopathy (BSE) in cattle [2–5] led to global concern that human exposure to BSE prions posed a significant threat to public health [6–8]. While the risk of new dietary exposure to BSE prions in the UK is now remote [9], the majority of the UK population may have been exposed during the late 1980s and early 1990s. While the number of recorded clinical cases (~200) has been relatively low, and epidemiological modelling argued against a large number of additional cases [10], it is increasingly clear that sub-clinical carrier states of prion infection may occur, particularly on crossing a species barrier and with low-dose exposure [11–15]. Modelling cannot estimate the number of sub-clinically infected carriers, and the discrepancy between numbers of clinical cases and prevalence estimates from

population screening remains unexplained [16–19]. Incubation periods in human prion infections, even in the absence of a species barrier, may exceed five decades [6,20,21], and multiple genetic loci in addition to the PrP gene are known to influence prion incubation periods in mice [22–25] and susceptibility in humans [26,27]. Asymptomatically infected carriers appear to have transmitted vCJD prion infection and disease to others via blood transfusion [28–30] or blood products [31]. Other iatrogenic routes, notably from contaminated surgical instruments, present a potential risk [6,8,32].

In the absence of a blood test for human prion disease, the distinct phenotype of vCJD has provided the basis for investigating infection prevalence within the UK population. The peripheral pathogenesis of vCJD differs significantly from that of classical CJD, inherited prion disease, and kuru, with disease-related PrP (PrP^{Sc}) being uniformly and prominently detected in a range of lymphoreticular tissues at autopsy [33–37]. To date, antemortem tonsil biopsy has shown 100% sensitivity and specificity for diagnosis of clinical vCJD [30,33,34], and the fact that lymphoreticular prion infection is not a feature of iatrogenic CJD [33,36] or kuru [21,38] argues that the distinct pathogenesis of vCJD relates to the effect of prion strain rather than to a peripheral route of infection [21,38,39]. Because lymphoreticular colonization is thought to precede neuroinvasion in vCJD, and indeed has been detected in archived surgical samples removed prior to the development of overt clinical symptoms [40,41], anonymous screening of lymphoreticular tissues removed during routine surgery appears to offer a practical means to estimate the population prevalence of asymptomatic vCJD infection. However, the sensitivity and specificity of such testing during the pre-clinical incubation period or in chronic carriers (who may have a distinct pathogenesis which conceivably might not involve the same degree of lymphoreticular colonization) are unknown. Although peripheral tissue involvement in recently identified cases of variable protease-sensitive prionopathy [42–45] has not yet been defined, the apparent rarity of this condition would not be expected to confound estimates of UK vCJD prevalence.

To date, three prevalence studies have been reported [41,46,47]. The first study used immunohistochemistry to retrospectively examine 11 247 appendicectomy and 1427 tonsillectomy specimens that had been archived as formalin-fixed tissue blocks [41]. Positive lymphoreticular accumulation of abnormal PrP was found in three appendixes from 10 278 specimens in a patient birth cohort of 1961–1985, giving a population prevalence in this age group of 292 (95% confidence interval 60–853) per million [41,47]. Subsequent studies have prospectively examined only tonsil as this tissue can be readily obtained from large numbers of routine tonsillectomy procedures. Tonsil appeared to be a more sensitive reporter of clinical vCJD infection than appendix in one study [48], although not in another [36]. The first prospective pilot scale study of 2000 specimens found no evidence for tonsillar accumulation of abnormal PrP using high sensitivity methods [46]. More recently, a much larger national study of 63 007 tonsils reported no positive specimens using enzyme immunoassays to detect disease-related PrP [47]. To confirm the reliability of this result, all specimens in the 1961–1985 birth cohort (9160 tonsils) plus controls were tested by immunohistochemistry [19,49]. Three specimens showed some initial reactivity, but for two this was concluded to be non-specific. For the third, one strongly positive follicle was identified on two slides from adjacent sections using different anti-PrP antibodies. However, subsequent re-sampling of this specimen revealed no further reactivity by either immunohistochemistry or immunoblotting [19,49]. Notably, an incidence of either zero or one positive in the 1961–1985 birth cohort in these large-scale studies of tonsil does not significantly alter the infection prevalence estimated by the original study of appendix specimens [19,47,49].

Inevitably, the failure to identify an unequivocally positive sample in more recent prospective studies has meant that the positive appendix specimens identified by Hilton *et al* have been the subject of considerable debate [17–19,41]. Central to this is whether the identified immuno-reactivity truly reflects authentic prion infection. Interpretation has been hampered by the inability to do confirmatory immunoblotting of formalin-fixed tissue and by the fact that two of the three positive specimens that could be analysed were from individuals with a *PRNP* codon 129 valine homozygous genotype [50]. Polymorphism at residue 129 of human PrP [encoding either methionine (M) or valine (V)] powerfully affects susceptibility to human prion diseases, with residue 129 acting to restrict the propagation of particular prion strains through conformational selection [6,51,52] and heterozygosity conferring resistance by inhibiting homologous protein–protein interactions [52–54]. To date, all patients with neuropathologically confirmed vCJD have been *PRNP* codon 129 methionine homozygotes [7,26] and have a remarkably uniform and distinct neuropathological phenotype defined by the presence of abundant florid PrP plaques [1] and the propagation of type 4 PrP^{Sc} [2,55] in the brain.

Transgenic modelling has provided a molecular explanation for these findings by showing that human PrP 129 valine is unable to propagate the vCJD prion strain [51] and that BSE- or vCJD-challenged human PrP 129V transgenic mice propagate novel prion strains that have not yet been identified in humans [3,39,51]. It is unclear whether humans of the *PRNP* 129VV genotype following infection with BSE prions would develop a clinical disease and if so, what clinicopathological phenotype would result [7,8,51]. Thus, in the absence of any natural history of BSE prion infection in humans with a *PRNP* codon 129VV genotype, no positive control tissue exists for comparison with the appendix specimens identified in the prevalence screen. A clinical vCJD case in a *PRNP*

codon 129 MV heterozygote has been reported, but no tissue biopsy or autopsy was performed [56]. In the present study, we have therefore sought to investigate these specimens further by attempting to transmit prion infection from formalin-fixed tissue to transgenic mice overexpressing human PrP. Owing to the paucity of tissue available, it was not possible to inoculate different lines of transgenic mice expressing either human PrP 129 methionine or valine. Based on our experience of the transmission properties of vCJD and BSE prions, we chose to use Tg45 mice homozygous for 129 methionine [5]. These mice faithfully propagate the molecular and neuropathological phenotype of vCJD following primary challenge with either vCJD prions [5,57] or BSE prions [5] and are known to be sensitive to infection when inoculated with vCJD peripheral tissue containing PrP^{Sc} at a concentration $10^{4.7}$-fold lower than in brain [57]. Importantly, the novel prion strain (associated with type 5 PrP^{Sc}) that is generated in vCJD-challenged human PrP 129V transgenic mice also transmits infection efficiently to transgenic mice expressing human PrP 129M [51].

Materials and methods

Ethical issues

Storage and biochemical analysis of human tissue samples and transmission studies to mice were performed with consent from patients or relatives under approval from the Local Research Ethics Committee of UCL Institute of Neurology/National Hospital for Neurology and Neurosurgery and the code of practice specified in the Human Tissue Authority licence held by UCL Institute of Neurology. Work with mice was performed under licence granted by the UK Home Office and conformed to University College London institutional guidelines. All procedures were carried out in microbiological containment level 3 facilities with strict adherence to safety protocols.

Preparation of inocula

Frozen brain (frontal cortex) and appendix (full thickness tissue) from a single neuropathologically confirmed vCJD patient [48] were prepared as 10% w/v homogenates in Dulbecco's sterile phosphate-buffered saline lacking Ca^{2+} and Mg^{2+} ions (D-PBS) by either serial passage through needles of decreasing diameter (brain) or the use of a Duall tissue grinder (appendix). PrP^{Sc}-positive 10% w/v homogenates [48] were diluted to 1% w/v with D-PBS and passed through a 25-gauge syringe needle prior to storage at −70 °C. Formalin-fixed, paraffin-embedded (FFPE) tissues that had not been exposed to formic acid were each available as three tissue sections each of approximate dimensions 4 µm × 0.5 cm × 1 cm mounted on glass slides. FFPE tissues for investigation comprised vCJD brain and vCJD spleen and two of the three abnormal PrP-positive appendix specimens (both PRNP codon 129 valine homozygous genotype) identified in the prevalence screen by Hilton *et al* [41]. Tissue was recovered by sequential treatment, firstly to remove paraffin by immersion in two changes of xylene for 5 min, followed by sequential immersion for 5 min in graded ethanol (100% × 2 and 70% × 1), after which tissue was re-hydrated by immersion in D-PBS for 5 min. Each of the cognate tissue sections was scraped from the glass slides and pooled in 300 µl of D-PBS and passaged through 16-, 19-, 21-, and 23-gauge syringe needles prior to storage at −70 °C. We estimate that this procedure produced tissue homogenates of a concentration of ∼0.2% w/v.

Transmission studies

Transgenic mice homozygous for a human PrP 129 methionine (M) transgene array and murine PrP null alleles ($Prnp^{o/o}$) designated Tg(HuPrP129M$^{+/+}$ $Prnp^{o/o}$)-45 (129 MM Tg45 mice) have been described previously [5,57,58]. Inocula (30 µl of tissue homogenate in D-PBS as described above) were injected intracerebrally into groups of ten 129 MM Tg45 mice as described elsewhere [5] and thereafter examined daily and were killed if they exhibited signs of distress or once a clinical diagnosis of prion disease was established. Brains from inoculated mice were analysed by high sensitivity immunoblotting and by neuropathological examination.

Immunohistochemistry

Transgenic mouse brain was analysed with anti-PrP monoclonal antibody ICSM 35 (D-Gen Ltd, London, UK), using a Ventana automated immunohistochemical staining machine (Ventana Medical Systems Inc, Tucson, AZ, USA) as described previously [38,59]. Briefly, tissue was fixed in 10% buffered formal saline followed by incubation in 98% formic acid for 1 h. Following further washing for 24 h in 10% buffered formal saline, tissue samples were processed and paraffin wax-embedded. Sections were cut at a nominal thickness of 4 µm, treated with 98% formic acid for 5 min, and then boiled in a low ionic strength buffer (2.1 mM Tris, 1.3 mM EDTA, 1.1 mM sodium citrate, pH 7.8) for 20 min. Abnormal PrP accumulation was examined using anti-PrP monoclonal ICSM 35 followed by a biotinylated anti-mouse IgG secondary antibody (*i*View Biotinylated Ig, Ventana Medical Systems Inc) and an avidin–biotin horseradish peroxidase conjugate (*i*View SA-HRP, Ventana Medical Systems Inc) before development with 3′,3-diaminobenzidine tetrachloride as the chromogen (*i*View DAB, Ventana Medical Systems Inc). Haematoxylin was used as the counterstain. Haematoxylin and eosin staining of serial sections was performed using conventional methods [59]. Appropriate controls were used throughout.

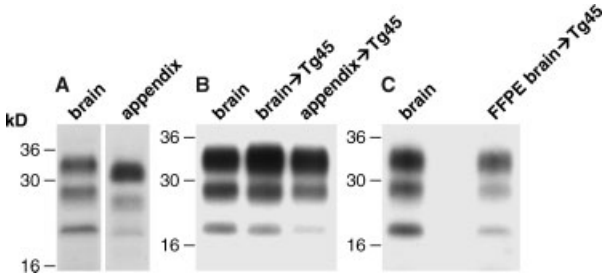

Figure 1. Molecular strain typing of vCJD brain and appendix transmissions in transgenic mice. (A–C) Immunoblots of proteinase-K-treated homogenates of vCJD tissues or 129 MM Tg45 transgenic mouse brain analysed by enhanced chemiluminescence with anti-PrP monoclonal antibody 3F4. All brain samples were analysed directly (between 1 and 8 µl of 10% w/v homogenate). vCJD appendix was pre-concentrated with sodium phosphotungstic acid from 500 µl of 10% w/v homogenate. (A) vCJD brain and appendix. Densitometry of this blot showed that the relative proportion of di-glycosylated PrP in PrP^{Sc} in the brain or appendix was 46% or 74%, respectively. (B) vCJD brain compared with 129 MM Tg45 mouse brain after transmission of vCJD brain or appendix. (C) vCJD brain compared with 129 MM Tg45 mouse brain after transmission of formalin-fixed, paraffin-embedded (FFPE) vCJD brain.

Table 1. Summary of transmissions of human tissues to 129 MM Tg45 transgenic mice

Inoculum*	Clinical signs	Incubation period (days ± SEM)	Total attack rate†
vCJD brain	4/10	396 ± 22	9/10
vCJD appendix	1/7	457	4/7
FFPE vCJD brain	3/9	580 ± 21	5/9
FFPE vCJD spleen	0/8	>359‡	0/8
FFPE prevalence appendix 1	0/4	>609	0/4
FFPE prevalence appendix 2	0/8	>405§	0/8

*Mice were inoculated with 30 µl of either 1% w/v vCJD brain or vCJD appendix homogenate, or ~0.2% w/v formalin-fixed, paraffin-embedded (FFPE) tissue homogenate. †Total attack rate is defined as the total number of both clinically affected and sub-clinically infected mice as a proportion of the number of inoculated mice. Sub-clinical prion infection was assessed by analysis for PrP^{Sc} (by either direct immunoblotting or after sodium phosphotungstic acid precipitation of 250 µl of 10% w/v brain homogenate) and also by immunohistochemical examination of brain. ‡Mice were culled at 360, 442, 442, 610, 610, 610, 610, and 610 days post-inoculation. §Mice were culled at 406, 451, 561, 610, 610, 610, 610, and 610 days post-inoculation.

Immunoblotting

Human or transgenic mouse tissues prepared as 10% w/v homogenates in D-PBS were analysed by proteinase K digestion (50 or 100 µg/ml final protease concentration, 1 h, 37 °C) and immunoblotting with anti-PrP monoclonal antibody 3F4 [60] using high sensitivity enhanced chemiluminescence as described previously [34,59]. For analysis of PrP glycoforms, blots were developed in chemifluorescent substrate (AttoPhos; Promega, Madison, WI, USA) and visualized on a Storm 840 phosphoimager (GE Healthcare, Little Chalfont, UK) using ImageQuaNT software (GE Healthcare) [34,61]. Sodium phosphotungstic acid precipitation of PrP^{Sc} from tissue homogenate [62] was performed as described previously [34,59].

Results

Previously we have reported the comparison of PrP^{Sc} in brain and appendix from the same vCJD patient [48]. PrP^{Sc} in appendix was at a concentration ~0.5% of that present in brain [48] and was distinct from type 4 PrP^{Sc} in brain, being characterized by a greater predominance of di-glycosylated PrP (Figure 1A) closely similar to that seen in tonsil and other lymphoreticular tissues in vCJD [33,34,36,37,57]. These PrP^{Sc}-positive tissue homogenates were used as positive controls for transmission studies in comparison to FFPE tissue specimens. Consistent with our previous findings [5,57], intra-cerebral challenge of 129 MM Tg45 transgenic mice with vCJD brain homogenate resulted in efficient transmission of prion infection, with nine of ten inoculated mice scored as affected (Table 1). Four mice developed clinical prion disease with a mean incubation period of 396 ± 22 days, while five other recipients were sub-clinically infected (Table 1). The single mouse scored as non-affected was culled, due to inter-current illness at 280 days post-inoculation. All nine infected mice had detectable PrP^{Sc} in brain that showed a predominance of di-glycosylated PrP, and accurate molecular strain typing was possible with four samples, which confirmed the propagation of type 4 PrP^{Sc} (Figure 1B). In two of these brains, the presence of abundant florid PrP plaques was demonstrated by immunohistochemistry (Figure 2).

In comparison, and consistent with a lower concentration of PrP^{Sc} in the inoculum, vCJD appendix transmitted infection to 129 MM Tg45 mice with lower efficiency. Four of seven inoculated mice were infected, but only a single mouse developed clinical prion disease at 457 days post-inoculation (Table 1). The three mice scored as non-affected were culled between 314 and 395 days post-inoculation. All four infected mice had detectable PrP^{Sc} in brain that showed a predominance of di-glycosylated PrP, and three of these samples had PrP^{Sc} at sufficient concentration to permit accurate molecular strain typing. This analysis showed the propagation of type 4 PrP^{Sc} with no statistically significant difference in the PrP^{Sc} glycoform ratio from that seen in mice inoculated with vCJD brain (Table 2). All three vCJD appendix-inoculated 129 MM Tg45 mice propagating high levels of type 4 PrP^{Sc} showed the presence of florid PrP plaques in the brain by immunohistochemistry (Figure 2). Thus, despite a difference in the glycoform ratio of PrP^{Sc} between vCJD brain and appendix (Figure 1A), congruent transmission of the vCJD prion strain was seen in human PrP 129M transgenic mice characterized by the propagation of type 4 PrP^{Sc} and the occurrence of florid PrP plaques in the brain.

While transmission of sporadic Creutzfeldt–Jakob disease prions from formalin-fixed human brain tissue to primates has been reported [63,64], to date there have been no data concerning the effect of formaldehyde on vCJD prions. We found that FFPE

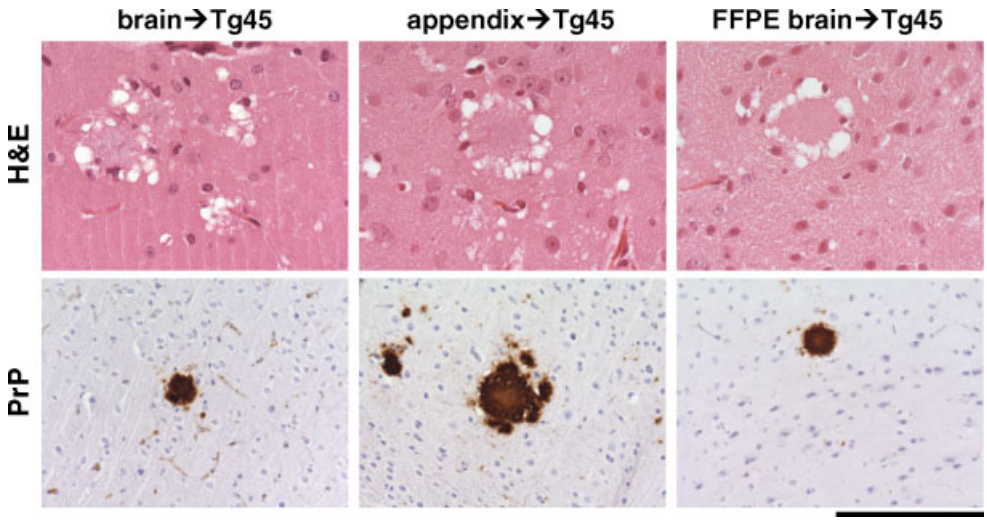

Figure 2. Neuropathological analysis of transgenic mouse brain. Transmission of vCJD brain homogenate, vCJD appendix homogenate, and formalin-fixed, paraffin-embedded (FFPE) vCJD brain to 129 MM Tg45 mice. Haematoxylin- and eosin-stained sections (H&E) show spongiform neurodegeneration in the cortex, including florid plaques that show abnormal PrP immunoreactivity (PrP) stained with anti-PrP monoclonal antibody ICSM 35. Scale bar: H&E, 40 μm; PrP, 100 μm.

Table 2. Glycoform analysis of type 4 PrP^{Sc} propagated in the brains of 129 MM Tg45 mice after transmission of vCJD brain or appendix

PrP glycoform*	Transmission from brain	Transmission from appendix	*p* value†
Di-glycosylated	55 ± 2	53 ± 3	0.15
Mono-glycosylated	30 ± 1	32 ± 3	0.23
Non-glycosylated	15 ± 2	15 ± 1	0.75

*Relative proportion of each PrP glycoform (mean ± SD %) in type 4 PrP^{Sc} propagated in the brains of 129 MM Tg45 transgenic mice inoculated with vCJD brain ($n = 4$ mice) or appendix ($n = 3$ mice). †Unpaired two-tailed *t*-test.

vCJD brain transmitted infection to five of nine inoculated 129 MM Tg45 mice without an apparent change in prion strain properties. Three inoculated mice developed clinical prion disease, with a mean incubation period of 580 ± 21 days, while two other recipients were sub-clinically infected (Table 1). The four mice scored as non-affected were culled between 540 and 610 days post-inoculation. All five infected mice had positive PrP immunohistochemistry, and in three brains prominent florid PrP plaques were observed (Figure 2). Accurate molecular strain typing was possible with two brains, which confirmed the propagation of type 4 PrP^{Sc} (Figure 1C).

Although the strain properties of vCJD prions are preserved after exposure to formaldehyde, the unknown level of prion infectivity present in the starting vCJD brain tissue prevents us from commenting on how formalin fixation and subsequent processing may have affected vCJD prion titre. However, the reduced attack rate and more prolonged mean incubation period observed with the FFPE vCJD brain compared with the control vCJD brain homogenate are consistent with a significantly reduced prion titre. In this regard, formalin fixation of prion-infected hamster brain followed by histological processing in the absence of exposure to formic acid has been reported to reduce prion infectivity between 2 and 3 logs [65,66].

In contrast to FFPE vCJD brain, FFPE vCJD spleen and both of the FFPE abnormal PrP-positive appendix specimens failed to transmit prion infection to 129 MM Tg45 mice (Table 1). Exhaustive examination of brains from mice at up to 610 days post-inoculation failed to reveal the presence of PrP^{Sc} after sodium phosphotungstic acid precipitation or the presence of abnormal PrP deposition by immunohistochemistry (data not shown).

Discussion

Current UK Department of Health risk assessment and risk management are based to a large extent on the results of the Hilton *et al* study [17–19]. Fundamental to this is whether the three positives identified by PrP immunohistochemistry are 'true positives' indicating vCJD prion infection. In this study, we have attempted to transmit prion infectivity from FFPE tissue sections derived from two abnormal PrP-positive appendix specimens identified in the retrospective vCJD prevalence screen of Hilton *et al* [41]. While transgenic mice expressing human PrP 129M reported vCJD prion infection after inoculation with frozen vCJD brain or appendix and also FFPE vCJD brain, no prion transmission was observed from FFPE vCJD spleen or the FFPE appendix specimens. However, these negative findings cannot be interpreted, as the levels of infectivity in the positive control lymphoreticular tissue following formalin fixation were below the detection limit of our assay. It is entirely possible, therefore, that the Hilton *et al* positives were indeed prion-infected and that a lower titre in these pre-clinical tissues and/or the effect of formalin fixation precluded detection by our

bioassay. Unless more sensitive assays for vCJD prions become available, verification of the estimated UK prevalence of vCJD infection will have to come from future prevalence studies [17–19,47].

Although the present findings are not informative with regard to vCJD prevalence, two results are of some importance. Firstly, we have demonstrated that the strain properties of vCJD prions do not appear to be affected by tissue fixation with formaldehyde or by exposure to other chemicals that are commonly used during histological processing. Secondly, we have shown that prions associated with distinct PrP^{Sc} glycotypes in vCJD brain (type 4) [2] or lymphoreticular tissues (type 4t) [33,34] do not transmit disease with distinct prion strain properties. These findings are in agreement with two other recent studies that have also shown congruent prion strain properties after transmission of brain or lymphoid tissue from sheep scrapie to bank voles [67] or from vCJD to wild-type mice [68]. Prion strains may comprise an ensemble or quasi-species maintained under selection pressure in a host and the populations of which may therefore differ in different tissues [52,69]. According to this general model [52], even if a distinct prion strain were propagating in lymphoreticular as opposed to CNS tissue, it would revert to the brain-specific strain type on passage in transgenic mouse brain. Thus, based on the current findings and other published data [30,70] it appears that molecular and neuropathological examination will not be able to differentiate between primary vCJD cases resulting from exposure to BSE prions and secondary cases resulting from iatrogenic exposure to vCJD-infected peripheral tissues.

Acknowledgment

We thank all patients and their families for generously consenting to the use of human tissues in this research. This work was funded by the Medical Research Council. Some of the work was undertaken at University College London Hospital NHS Foundation Trust, which received a proportion of funding from the Department of Health's NIHR Biomedical Research Centres funding scheme.

Author contribution statement

JDFW and JC coordinated the design and operation of the study, and drafted the manuscript. JWI and DAH identified the positive appendix specimens, provided formalin-fixed tissues, and contributed to the design of the study. EAA contributed to the study design and generated transgenic mice. JDFW, ID-M, and SJ performed the biochemical analysis. JL, CO'M, CP, and SB carried out neuropathological analysis. All authors contributed to and approved the final version of the manuscript. JC had full access to all the data in the study and had final responsibility for the decision to submit for publication.

References

1. Will RG, Ironside JW, Zeidler M, *et al*. A new variant of Creutzfeldt–Jakob disease in the UK. *Lancet* 1996; **347**: 921–925.
2. Collinge J, Sidle KC, Meads J, *et al*. Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature* 1996; **383**: 685–690.
3. Hill AF, Desbruslais M, Joiner S, *et al*. The same prion strain causes vCJD and BSE. *Nature* 1997; **389**: 448–450, 526.
4. Bruce ME, Will RG, Ironside JW, *et al*. Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature* 1997; **389**: 498–501.
5. Asante EA, Linehan JM, Desbruslais M, *et al*. BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. *EMBO J* 2002; **21**: 6358–6366.
6. Collinge J. Variant Creutzfeldt–Jakob disease. *Lancet* 1999; **354**: 317–323.
7. Collinge J. Molecular neurology of prion disease. *J Neurol Neurosurg Psychiatry* 2005; **76**: 906–919.
8. Wadsworth JD, Collinge J. Update on human prion disease. *Biochim Biophys Acta* 2007; **1772**: 598–609.
9. Adkin A, Webster V, Arnold ME, *et al*. Estimating the impact on the food chain of changing bovine spongiform encephalopathy (BSE) control measures: the BSE control model. *Prev Vet Med* 2010; **93**: 170–182.
10. Ghani AC, Donnelly CA, Ferguson NM, *et al*. Updated projections of future vCJD deaths in the UK. *BMC Infect Dis* 2003; **3**: 4.
11. Hill AF, Joiner S, Linehan J, *et al*. Species barrier independent prion replication in apparently resistant species. *Proc Natl Acad Sci U S A* 2000; **97**: 10248–10253.
12. Hill AF, Collinge J. Subclinical prion infection in humans and animals. *Br Med Bull* 2003; **66**: 161–170.
13. Race R, Raines A, Raymond GJ, *et al*. Long-term subclinical carrier state precedes scrapie replication and adaptation in a resistant species: analogies to bovine spongiform encephalopathy and variant Creutzfeldt–Jakob disease in humans. *J Virol* 2001; **75**: 10106–10112.
14. Thackray AM, Klein MA, Aguzzi A, *et al*. Chronic subclinical prion disease induced by low-dose inoculum. *J Virol* 2002; **76**: 2510–2517.
15. Georgsson G, Adolfsdottir JA, Palsdottir A, *et al*. High incidence of subclinical infection of lymphoid tissues in scrapie-affected sheep flocks. *Arch Virol* 2008; **153**: 637–644.
16. Clarke P, Ghani AC. Projections of the future course of the primary vCJD epidemic in the UK: inclusion of subclinical infection and the possibility of wider genetic susceptibility. *J R Soc Interface* 2005; **2**: 19–31.
17. SEAC. SEAC 100/6. Resolving the discrepancy in current estimates. Spongiform Encephalopathy Advisory Committee; [Accessed 13 September 2010]: Available from: http://www.seac.gov.uk/papers/paper100-6.pdf.
18. SEAC. SEAC 100/2. Combining evidence from tissue surveys to estimate prevalence of subclinical vCJD. Spongiform Encephalopathy Advisory Committee; [Accessed 13 September 2010]: Available from: http://www.seac.gov.uk/papers/paper100-2.pdf.
19. SEAC. SEAC 104/2. Estimating the prevalence of subclinical vCJD. Spongiform Encephalopathy Advisory Committee; [Accessed 13 September 2010]: Available from: http://www.seac.gov.uk/papers/104-2.pdf.
20. Collinge J, Whitfield J, McKintosh E, *et al*. Kuru in the 21st century—an acquired human prion disease with very long incubation periods. *Lancet* 2006; **367**: 2068–2074.
21. Collinge J, Whitfield J, McKintosh E, *et al*. A clinical study of kuru patients with long incubation periods at the end of the

epidemic in Papua New Guinea. *Philos Trans R Soc London Ser B Biol Sci* 2008; **363**: 3725–3739.
22. Stephenson DA, Chiotti K, Ebeling C, *et al*. Quantitative trait loci affecting prion incubation time in mice. *Genomics* 2000; **69**: 47–53.
23. Lloyd S, Onwuazor ON, Beck J, *et al*. Identification of multiple quantitative trait loci linked to prion disease incubation period in mice. *Proc Natl Acad Sci U S A* 2001; **98**: 6279–6283.
24. Lloyd S, Uphill JB, Targonski PV, *et al*. Identification of genetic loci affecting mouse-adapted bovine spongiform encephalopathy incubation time in mice. *Neurogenetics* 2002; **4**: 77–81.
25. Lloyd SE, Maytham EG, Grizenkova J, *et al*. A Copine family member, Cpne8, is a candidate quantitative trait gene for prion disease incubation time in mouse. *Neurogenetics* 2009; **11**: 185–191.
26. Mead S, Poulter M, Uphill J, *et al*. Genetic risk factors for variant Creutzfeldt–Jakob disease: a genome-wide association study. *Lancet Neurol* 2009; **8**: 57–66.
27. Lloyd SE, Maytham EG, Pota H, *et al*. HECTD2 is associated with susceptibility to mouse and human prion disease. *PLoS Genet* 2009; **5**: e1000383.
28. Llewelyn CA, Hewitt PE, Knight RS, *et al*. Possible transmission of variant Creutzfeldt–Jakob disease by blood transfusion. *Lancet* 2004; **363**: 417–421.
29. Peden AH, Head MW, Ritchie DL, *et al*. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. *Lancet* 2004; **364**: 527–529.
30. Wroe SJ, Pal S, Siddique D, *et al*. Clinical presentation and premortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: a case report. *Lancet* 2006; **368**: 2061–2067.
31. Peden A, McCardle L, Head MW, *et al*. Variant CJD infection in the spleen of a neurologically asymptomatic UK adult patient with haemophilia. *Haemophilia* 2010; **16**: 296–304.
32. Armitage WJ, Tullo AB, Ironside JW. Risk of Creutzfeldt–Jakob disease transmission by ocular surgery and tissue transplantation. *Eye* 2009; **10**: 1926–1930.
33. Hill AF, Butterworth RJ, Joiner S, *et al*. Investigation of variant Creutzfeldt–Jakob disease and other human prion diseases with tonsil biopsy samples. *Lancet* 1999; **353**: 183–189.
34. Wadsworth JD, Joiner S, Hill AF, *et al*. Tissue distribution of protease resistant prion protein in variant CJD using a highly sensitive immuno-blotting assay. *Lancet* 2001; **358**: 171–180.
35. Hilton DA, Sutak J, Smith ME, *et al*. Specificity of lymphoreticular accumulation of prion protein for variant Creutzfeldt–Jakob disease. *J Clin Pathol* 2004; **57**: 300–302.
36. Head MW, Ritchie D, Smith N, *et al*. Peripheral tissue involvement in sporadic, iatrogenic, and variant Creutzfeldt–Jakob disease: an immunohistochemical, quantitative, and biochemical study. *Am J Pathol* 2004; **164**: 143–153.
37. Joiner S, Linehan J, Brandner S, *et al*. High levels of disease related prion protein in the ileum in variant Creutzfeldt–Jakob disease. *Gut* 2005; **54**: 1506–1508.
38. Brandner S, Whitfield J, Boone K, *et al*. Central and peripheral pathology of kuru: pathological analysis of a recent case and comparison with other forms of human prion disease. *Philos Trans R Soc London Ser B Biol Sci* 2008; **363**: 3755–3763.
39. Wadsworth JD, Joiner S, Linehan JM, *et al*. Kuru prions and sporadic Creutzfeldt–Jakob disease prions have equivalent transmission properties in transgenic and wild-type mice. *Proc Natl Acad Sci U S A* 2008; **105**: 3885–3890.
40. Hilton DA, Fathers E, Edwards P, *et al*. Prion immunoreactivity in appendix before clinical onset of variant Creutzfeldt–Jakob disease. *Lancet* 1998; **352**: 703–704.
41. Hilton DA, Ghani AC, Conyers L, *et al*. Prevalence of lymphoreticular prion protein accumulation in UK tissue samples. *J Pathol* 2004; **203**: 733–739.
42. Gambetti P, Dong Z, Yuan J, *et al*. A novel human disease with abnormal prion protein sensitive to protease. *Ann Neurol* 2008; **63**: 697–708.
43. Zou WQ, Puoti G, Xiao X, *et al*. Variably protease-sensitive prionopathy: a new sporadic disease of the prion protein. *Ann Neurol* 2010; **68**: 162–172.
44. Jansen C, Head MW, Van Gool WA, *et al*. The first case of protease-sensitive prionopathy (PSPr) in The Netherlands: a patient with an unusual GSS-like clinical phenotype. *J Neurol Neurosurg Psychiatry* 2010; **81**: 1052–1055.
45. Head MW, Knight R, Zeidler M, *et al*. A case of protease sensitive prionopathy in a patient in the United Kingdom. *Neuropathol Appl Neurobiol* 2009; **35**: 628–632.
46. Frosh A, Smith LC, Jackson CJ, *et al*. Analysis of 2000 consecutive UK tonsillectomy specimens for disease-related prion protein. *Lancet* 2004; **364**: 1260–1262.
47. Clewley JP, Kelly CM, Andrews N, *et al*. Prevalence of disease related prion protein in anonymous tonsil specimens in Britain: cross sectional opportunistic survey. *Br Med J* 2009; **338**: b1442.
48. Joiner S, Linehan J, Brandner S, *et al*. Irregular presence of abnormal prion protein in appendix in variant Creutzfeldt–Jakob disease. *J Neurol Neurosurg Psychiatry* 2002; **73**: 597–598.
49. de Marco MF, Linehan J, Gill ON, *et al*. Large-scale immunohistochemical examination for lymphoreticular prion protein in tonsil specimens collected in Britain. *J Pathol* 2010; DOI:10.1002/path.2767.
50. Ironside JW, Bishop MT, Connolly K, *et al*. Variant Creutzfeldt–Jakob disease: prion protein genotype analysis of positive appendix tissue samples from a retrospective prevalence study. *Br Med J* 2006; **2006**: 1164–1165.
51. Wadsworth JD, Asante EA, Desbruslais M, *et al*. Human prion protein with valine 129 prevents expression of variant CJD phenotype. *Science* 2004; **306**: 1793–1796.
52. Collinge J, Clarke A. A general model of prion strains and their pathogenicity. *Science* 2007; **318**: 930–936.
53. Collinge J, Palmer MS, Dryden AJ. Genetic predisposition to iatrogenic Creutzfeldt–Jakob disease. *Lancet* 1991; **337**: 1441–1442.
54. Palmer MS, Dryden AJ, Hughes JT, *et al*. Homozygous prion protein genotype predisposes to sporadic Creutzfeldt–Jakob disease. *Nature* 1991; **352**: 340–342.
55. Hill AF, Joiner S, Wadsworth JD, *et al*. Molecular classification of sporadic Creutzfeldt–Jakob disease. *Brain* 2003; **126**: 1333–1346.
56. Kaski D, Mead S, Hyare H, *et al*. Variant CJD in an individual heterozygous for PRNP codon 129. *Lancet* 2009; **374**: 2128.
57. Wadsworth JD, Joiner S, Fox K, *et al*. Prion infectivity in variant Creutzfeldt–Jakob disease rectum. *Gut* 2007; **56**: 90–94.
58. Asante E, Linehan J, Gowland I, *et al*. Dissociation of pathological and molecular phenotype of variant Creutzfeldt–Jakob disease in transgenic human prion protein 129 heterozygous mice. *Proc Natl Acad Sci U S A* 2006; **103**: 10759–10764.
59. Wadsworth JD, Powell C, Beck J, *et al*. Molecular diagnosis of human prion disease. *Methods Mol Biol* 2008; **459**: 197–227.
60. Kascsak RJ, Rubenstein R, Merz PA, *et al*. Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. *J Virol* 1987; **61**: 3688–3693.
61. Hill AF, Joiner S, Beck J, *et al*. Distinct glycoform ratios of protease resistant prion protein associated with *PRNP* point mutations. *Brain* 2006; **129**: 676–685.
62. Safar J, Wille H, Itri V, *et al*. Eight prion strains have PrP^{Sc} molecules with different conformations. *Nature Med* 1998; **4**: 1157–1165.

63. Brown P, Gibbs CJ Jr, Gajdusek DC, *et al*. Transmission of Creutzfeldt–Jakob disease from formalin-fixed, paraffin-embedded human brain tissue. *N Engl J Med* 1986; **315**: 1614–1615.
64. Brown P, Gibbs CJ Jr, Rodgers Johnson P, *et al*. Human spongiform encephalopathy: the National Institutes of Health series of 300 cases of experimentally transmitted disease. *Ann Neurol* 1994; **35**: 513–529.
65. Brown P, Rohwer RG, Green EM, *et al*. Effect of chemicals, heat, and histopathologic processing on high-infectivity hamster-adapted scrapie virus. *J Infect Dis* 1982; **145**: 683–687.
66. Brown P, Liberski PP, Wolff A, *et al*. Resistance of scrapie infectivity to steam autoclaving after formaldehyde fixation and limited survival after ashing at 360 °C: practical and theoretical implications. *J Infect Dis* 1990; **161**: 467–472.
67. Di Bari MA, Chianini F, Vaccari G, *et al*. The bank vole (*Myodes glareolus*) as a sensitive bioassay for sheep scrapie. *J Gen Virol* 2008; **89**: 2975–2985.
68. Ritchie DL, Boyle A, McConnell I, *et al*. Transmissions of variant Creutzfeldt–Jakob disease from brain and lymphoreticular tissue show uniform and conserved BSE-related phenotypic properties on primary and secondary passage in wild-type mice. *J Gen Virol* 2009; **90**: 3075–3082.
69. Li J, Browning S, Mahal SP, *et al*. Darwinian evolution of prions in cell culture. *Science* 2010; **327**: 869–872.
70. Bishop MT, Ritchie DL, Will RG, *et al*. No major change in vCJD agent strain after secondary transmission via blood transfusion. *PLoS ONE* 2008; **3**: e2878.